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Short communication

Proteomics inference of genes involved in host adaptation of *Mycoplasma gallinarum*

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ABSTRACT

Different from most other host-specific mycoplasmas, *Mycoplasma gallinarum* has been isolated from various hosts, such as poultry, pig, cattle, and sheep. The wide distribution among different hosts, the low pathogenesis, and the weak host immunological responses suggest this mycoplasma has a unique host adaptation mechanism. In this study, we applied two-dimensional liquid chromatography electrospray ionization tandem mass spectrometry (2D LC–MS/MS) to characterize the protein profiling of *M. gallinarum*. Our results suggest that *M. gallinarum* possesses homologs of cytadhesin proteins found in other mycoplasmas lacking an organized tip organelle. Our results showed that there are possibly multiple aminopeptidase gene homologs present in *M. gallinarum*, which might be involved in nutrient acquisition of *M. gallinarum*. The information present here would be useful for future studies to identify genes responsible for the colonization and host adaptation properties of *M. gallinarum*.

1. Introduction

Different from other host-specific mycoplasmas, *Mycoplasma gallinarum* has been isolated from various hosts, including poultry, swine, cattle, and sheep (Kleven et al., 1978; Shah-Majid and Nihayah, 1987; Poveda et al., 1990a,b; Singh and Uppal, 1987; Taylor-Robinson and Dinter, 1968). Generally, *M. gallinarum* alone does not cause any symptoms or lesions among infected hosts (Power and Jordan, 1976; Varley and Jordan, 1978a,b; Shah-Majid, 1994). Inoculation of chickens with *M. gallinarum* may cause airsacculitis when combined with infection by infectious bronchitis virus or vaccination for Newcastle disease or infectious bronchitis (Kleven, 1998; Kleven et al., 1978; Shah-Majid, 1996). In contrast to more virulent poultry mycoplasmas, *M. gallinarum* generally induces a weak humoral immune response in infected chickens (Bencina and Bradbury, 1991; Bencina et al., 1991). The wide distribution of *M. gallinarum* among different species of hosts and its persistent colonization demonstrates the presence of a unique host adaptation mechanism in this mycoplasma. The low pathogenesis of *M. gallinarum* and the weak host immunological responses suggest a unique mechanism to escape host immune responses.

However, little is known of the molecular mechanisms of host adaptation of *M. gallinarum*. Previously, we reported the distribution of leucyl aminopeptidase in *M. gallinarum* may be important for this bacterium to have a broad living environment (Wan et al., 2004a,b). The goal of this study is to isolate and identify proteins from *M. gallinarum* with 2D LC–MS/MS, through which tandem MS (MS2) first separates peptide based on mass and then in

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the second stage analysis the peptide will be fragmented to determine the amino acid sequence (Kinter and Sherman, 2000). This information would be useful for future studies to identify genes responsible for the colonization and host adaptation properties of this mycoplasma.

2. Materials and methods

2.1. Mycoplasma culture and 2D LC–MS/MS sample preparation

M. gallinarum cells were harvested from a 24 h 1-L culture by centrifugation at 3000 × *g* for 10 min, and then washed three times in phosphate buffered saline (PBS). The cells were then resuspended in 900 μL of PBS with 100 mM of phenylmethylsulfonyl fluoride and placed on ice. In order to reduce the complexity of protein extracts for 2D LC–MS/MS analysis, Triton® X-114 was used for phase partitioning of mycoplasma proteins as described previously (Wan et al., 2004b). The Triton® X-114 methodology separates proteins based on relative hydrophobicity (Wise et al., 1995) and was used as a method of prefractionation to reduce the complexity of the sample prior to mass spectrometry analysis. Briefly, Triton® X-114 was added to the suspension to a final concentration of 1% and the solution was incubated at 4 °C for 3.5 h. The solution was centrifuged at 13,000 × *g* at 4 °C for 1 min to remove the unsolubilized cells. The supernatants were collected and incubated at 37 °C for 10 min to partition the hydrophobic and hydrophilic phases, and then centrifuged at 2000 × *g* for 5 min. The aqueous phase was the hydrophilic fraction. The pellet was partitioned an additional time by adding the equal amount of water and then mixed briefly. The final lower phase was the hydrophobic phase of *M. gallinarum* cells. Duplicate samples were prepared to increase the experimental accuracy.

2.2. Trypsin digestion and 2D LC ESI MS

For each replicate of each phase (membrane phase, cytosolic phase, or total protein), equal amounts of protein were precipitated with 25% trichloroacetic acid to remove salts and detergents. Protein pellets were resuspended in 0.1 M ammonium bicarbonate, 5% HPLC grade acetonitrile and reduced with 5 mM DTT for 5 min at 65 °C, and then alkylated with 10 mM iodoacetamide for 30 min at 30 °C. Proteins were then digested with trypsin at an enzyme/substrate ratio of 1:50 (w/w) at 37 °C for 16 h. Peptides were desalted using a peptide microtrap (Michrom BioResources, Inc., Auburn, CA) and eluted using a 0.1% trifluoroacetic acid, 95% acetonitrile solution. Desalted peptides were dried in a vacuum centrifuge and resuspended in 20 μL of 0.1% formic acid for analysis by 2D LC–MS/MS as described (McCarthy et al., 2005).

2.3. Protein identification and data analysis

Mass spectra and tandem mass spectra were used to search subsets of the nonredundant protein database (nrpd) downloaded from GenBank using TurboSEQUEST (Bioworks Browser 3.2; ThermoElectron). For most of this work, we restricted our analyses to mycoplasmas species. A trypsin digestion data filter was applied *in silicon* to the database and mass changes due to cysteine carbamidomethylation and methionine oxidation. The peptide (MS precursor ion) mass tolerance was set to 1.5 Da and the fragment ion (MS) mass tolerance was set to 1.0 Da. Peptide matches were considered valid if they were more than seven amino acids with *X* correlation values of 1.5, 2.0 and 2.5 (+1, +2, and +3 ions, respectively) and Delta Cn values larger than 0.1. Only proteins present in both replicates in each phase (membrane, cytosolic, or total proteins) were considered a positive protein hit.

In order to lower the potential false positive rate, we grouped the proteins in three categories: (1) membrane phase and total proteins (TM); (2) cytosolic phase and total protein (TC); (3) cytosolic and membrane phase and total proteins (TMC). The proteins present in cytosolic or membrane phases but not in total protein will not be discussed in this study.

The functional categories for each gene were downloaded from JCVI Microbe comprehensive database (http://cmr.jcvi.org/tigr-scripts/CMR/CmrHomePage.cgi). Each identified protein was classified into 17 functional categories. When no JCVI annotation was available, proteins were annotated manually based on literature searches and closely related homologs (determined by BLASTP). The locations of potential proteins in *M. gallinarum* were predicted by using the program SOSUI (Mitaku et al., 2002).

3. Results and discussion

In this study, we performed 2D LC–MS/MS for protein profiling of *M. gallinarum*. As a result, a total of 409 protein homologs for cytosolic phase, 193 for membrane phase, and 312 for total proteins were obtained (Fig. 1). The lower number of proteins identified in the whole cell lysate sample (total proteins) may result from the difficulty in identifying proteins of low abundance in a complex sample (Ramsby and Makowski, 1999). As shown in Fig. 1, TM has a number of 26 (6 as putative membrane proteins predicted by SOSUI), TC 123(18) and TMC 31(5) proteins. As shown in Fig. 2 and Table 1, the majority of the proteins

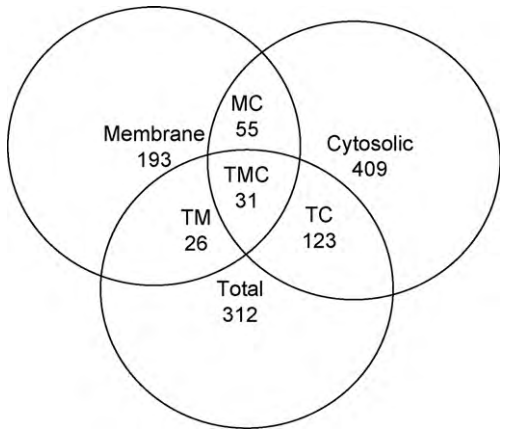

Fig. 1. Protein expression profiling of *Mycoplasma gallinarum*.

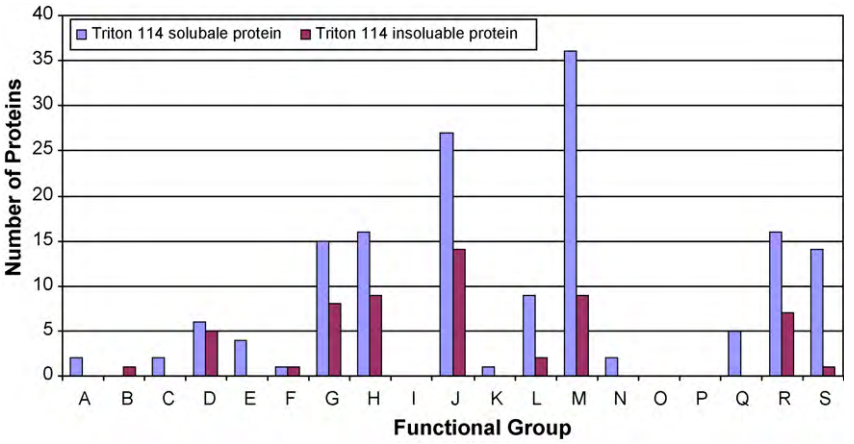

Fig. 2. Functional distribution of Triton®-114 treated proteins. Each bar represents the number of genes for the proteins identified either in soluble or insoluble phases. The genes are grouped in their corresponding functional and homology classes according to the JCVI annotation (http://cmr.jcvi.org/tigr-scripts/CMR/CmrHomePage.cgi): (A) amino acid biosynthesis; (B) biosynthesis of cofactors, prosthetic groups, and carriers; (C) cell division; (D) cell envelope; (E) cellular processes; (F) central intermediary metabolism; (G) DNA metabolism; (H) energy metabolism; (I) fatty acid and phospholipid metabolism; (J) hypothetical proteins; (K) mobile and extrachromosomal element function; (L) protein fate; (M) protein synthesis; (N) purines, pyrimidines, nucleosides, and nucleotides; (O) regulatory functions; (P) signal transduction; (Q) transcription; (R) transport and binding proteins, and (S) unknown function.

identified represent hypothetical proteins or proteins involved in protein synthesis.

Among the proteins we identified, a number of proteins are involved in DNA metabolism, transcription and protein synthesis. The DNA metabolism related proteins include DNA polymerase III, DNA or RNA helicase of superfamily II, DNA gyrase, NAD(+)-dependent DNA ligase, DNA glycosylases, endonuclease, excinuclease, recombinase, Mg-dependent DNAse, and ribose-phosphate pyrophosphokinase. RNA polymerase and transcription elongation factor NusA were also identified in both total membrane and cytosolic phases. The translation related genes include translation elongation factors (EF-TU and EF-G), 30S (S3, S5, S9, S7, S9, S13, and S19) and 50S (L4, L5, L6) ribosome subunits, various types of tRNA ligase, synthetase and tRNA amidotransferase. The common mycoplasmal chaperon protein DnaK, was also identified along with several heat shock proteins from *M. gallinarum*.

In addition to the proteins described above for DNA metabolism, transcription and protein synthesis, other predominant proteins that 2D LC–MS/MS identified are associated with cell adhesion and nutrition acquisition, and both of these functions are potentially responsible for the colonization and host adaptation of *M. gallinarum*.

3.1. Cytadhesins in M. gallinarum

Adhesion of mycoplasmas to host cells is a prerequisite for successful colonization and infection (Finlay and Falkow, 1997). The MS/MS data showed *M. gallinarum* has protein homologs of cytadherence high molecular weight protein 1 (HMW1) and 2 (HMW2) in *M. pneumonia*. *M. gallinarum* also has a protein homologous to the *M. hyopneumoniae* P97 protein, which functions as a heparin binding cytadhesin (Hsu et al., 1997). It was reported that P97 forms an operon with P102, both of which probably contribute to the adherence of *M. hyopneumoniae* (Hsu and Minion, 1998a,b). A P102 homolog was also identified in both membrane and cytosolic phases but not in the total protein (data not shown). *M. hyopneumoniae* does not have a specialized attachment organelle but P97 and P120 are both involved in the cell adherence (Adams et al., 2005; Hsu and Minion, 1998a,b). Additionally, a protein homologous to the *M. arthritidis* MAA1 protein was identified in the cytosolic protein fraction. The MAA1 protein functions as a major cytadhesin (Washburn et al., 2000; Bird et al., 2008), and the expression of a truncated version of the MAA1 protein reduced the virulence of *M. arthritis* (Washburn et al., 1993).

Therefore it is possible that *M. gallinarum* shares cytadhesin proteins with other mycoplasmas that lack a specialized tip organelle. Using transmission electron microscopic analysis, *M. gallinarum* was shown to have a circular shape without a specific attachment organelle (Dr. Mitch Balish, personal communication), similar to *M. hyopneumoniae*. Moreover, previous studies using cross-species hybridization with P30 and P1 probes failed to identify the P30 and P1 genes in *M. gallinarum* (Wan, 2002).

The fact that *M. gallinarum* expresses cytadhesin homologs from other mycoplasma species may explain, in part, the wide host range of this mycoplasma. It follows that the *M. gallinarum* cytadhesins could recognize a common glycoprotein(s) shared by different vertebrate animals. This kind of proposal was advanced in earlier studies to explain the proclivity of different mycoplasma species for the same host tissues (Leigh and Wise, 2002). Future studies will be required to address this possibility.

3.2. Versatile nutrition supplies in M. gallinarum

Our results showed that there are possibly five aminopeptidase gene homologs identified in *M. gallinarum*. Our earlier study demonstrated that *M. gallinarum* also shows a strong arginine aminopeptidase activity and relatively low leucine aminopeptidase activity (Wan, 2002). Leucyl aminopeptidases cleave leucine residues from the N-terminal of polypeptide chains, but substantial

Table 1
Protein identified in membrane and total proteins of *M. gallinarum* using 2D MS/MS.

Protein ID	Peptide hits (PH1/PH2)[a]			Species[b]	Location[c]	Gene function
	T	M	C			
Amino acid biosynthesis						
BAA04509	2/17		12/4	Unknown[d]	S	Ornithine transcarbamylase
NP_109994	2/8		7/4	MPM129	S	Ornithine transcarbamylase *(argl)*
Biosynthesis of cofactors, prosthetic groups, and carriers						
NP_975154	1/1	3/3		MMSC	S	Protoporphyrinogen oxidase
Cell division						
NP_072890	2/1		1/2	MGG37	S	Cell division protein *(ftsZ)*
YP_016237	1/1		1/2	MM163K	M (3)	Cell division protein *(ftsH)*
Cell envelope						
NP_975830	4/1	4/4		MMSC	M (8)	PTS system, glucose-specific IIBC component
NP_975843	4/1	4/4		MMSC	M (8)	PTS system, glucose-specific, IIBC component
AAF03943	2/2	1/1		MA	S	Putative adhesin *(MAA1)*
AAS00633	1/3		3/3	MHY	S	Adhesin *(P97)*
NP_110135	2/1		1/1	MPM129	S	Cytadherence accessory protein *(HMW1)*
NP_109998	**1/4**	**1/1**	**5/1**	**MPM129**	S	**Cytadherence accessory protein (HMW2)**
NP_072974	**2/1**	**3/3**	**3/1**	**MGG37**	**M (1)**	**Lipoprotein, putative**
YP_015810	1/3		2/5	MM163K	S	Chaperone protein *(DnaK)*
AAN85227	1/1		3/2	MF	M (2)	TraE/TrsE family NTPase
Cellular processes						
NP_325900	1/1		1/1	MPUAB	S	ATP-dependent RNA helicase
NP_326184	1/3		7/2	MPUAB	S	GTPase *(obgE, cgtA, yhbZ)*
NP_072684	1/5		1/1	MGG37	S	Translation-associated GTPase **(yChI-)**
AAD09235	2/1		2/1	MA	S	LicA homolog, mediating phase variation of lipooligosaccharide
Central intermediary metabolism						
AAN85227	1/1	1/1		MF	M (2)	TraE/TrsE family NTPase
YP_016218	2/1		3/1	MM163K	S	Inorganic pyrophosphatase
DNA metabolism						
AAT27773	**2/6**	**2/1**	**6/1**	**MM163K**	**S**	**Type I restriction-modification enzyme s subunit**
YP_015892	**1/1**	**2/2**	**2/1**	**MM163K**	**S**	**NAD(+)-dependent DNA ligase**
YP_016159	**2/2**	**1/1**	**2/2**	**MM163K**	**S**	**DNA or RNA helicase of superfamily II**
AAB37247	**2/1**	**1/1**	**1/2**	**MHO**	**S**	**DNA gyrase subunit A (gyrA)**
NP_325978	4/3		3/1	MPUAB	S	DNA gyrase subunit A *(gyrA)*
NP_757685	**1/2**	**3/3**	**2/1**	**MPHF2**	**S**	**Predicted MutT-like hydrolase**
NP_758210	1/1	1/1		MPHF2	S	Type I restriction-modification system M subunit
YP_016069	1/2	1/1		MM163K	S	Formamidopyrimidine-DNA glycosylase
NP_757587	1/5		4/1	MPHF2	S	Formamidopyrimidine-DNA glycosylase
NP_073092	1/2		2/2	MGG37	S	Excinuclease ABC subunit A *(uvrA)*
NP_109923	2/1		2/1	MPM129	S	Uracil-DNA glycosylase
NP_110016	2/2		1/1	MPM129	S	Endonuclease IV
NP_325926	1/2		1/1	MPUAB	S	6-O-methylguanine-DNA methyltransferase
NP_326446	3/5		6/2	MPUAB	S	Uracil-DNA glycosylase (udg)
NP_757678	1/2		1/4	MPHF2	S	Recombinase
NP_758303	1/4		5/5	MPHF2	S	DNA polymerase III subunit alpha
YP_016260	2/1		1/1	MM163K	S	Mg-dependent DNAse
NP_975919	4/1	4/6		MMSC	S	Ribose-phosphate pyrophosphokinase
Energy metabolism						
NP_853088	**5/2**	**1/1**	**3/2**	**MGR**	**S**	**ATP synthase subunit B**
YP_015905	**3/5**	**1/2**	**3/3**	**MM163K**	**S**	**ATP synthase subunit B**
YP_015993	**2/3**	**1/2**	**2/2**	**MM163K**	**S**	**ATP synthase subunit B**
NP_326281	2/2	1/1		MPUAB	S	ATP synthase subunit B
NP_757448	3/3	1/1		MPHF2	S	ATP synthase subunit B
NP_975598	4/2	2/1		MMSC	S	ATP synthase subunit B
NP_110287	3/2	1/1		MPM129	S	ATP synthase subunit B
NP_326097	3/1		1/4	MPUAB	S	ATP synthase subunit B
NP_326281	2/2		2/2	MPUAB	S	ATP synthase subunit B
NP_757448	3/3		2/3	MPHF2	S	ATP synthase subunit B
NP_975598	4/2		4/2	MMSC	S	ATP synthase subunit B
AAL34979	1/1		1/1	MHY	S	Pyruvate dehydrogenase El-beta subunit
NP_326594	17/6		13/4	MPUAB	S	Pyruvate dehydrogenase El-beta subunit
NP_073080	2/2	7/13		MGG37	S	Phosphopyruvate hydratase
NP_758322	1/1		3/1	MPHF2	S	Phosphoenolpyruvate carboxylase
YP_016008	**1/1**	**1/1**	**1/1**	**MM163K**	**S**	**Internal repeat-containing expressed protein**
NP_109770	1/1		2/1	MPM129	S	Transketolase 1

Table 1 (*Continued*)

Protein ID	Peptide hits (PH1/PH2)[a]			Species[b]	Location[c]	Gene function
	T	M	C			
NP_326428	4/27		22/21	MPUAB	S	3-Keto-L-gulonate-6-phosphate decarboxylase
NP_326488	2/3		2/1	MPUAB	S	Holliday junction DNA helicase motor protein
NP_758352	1/6		2/1	MPHF2	S	ROK family-glucose kinase or transcriptional regulator
NP_975491	1/1		1/1	MMSC	S	Glucose-6-phosphate isomerase
Hypothetical protein						
NP_325956	**1/3**	**2/1**	**4/3**	**MPUAB**	**M (1)**	**Hypothetical protein**
NP_326022	**1/1**	**1/1**	**1/1**	**MPUAB**	**M (1)**	**Hypothetical protein**
NP_326144	**1/2**	**2/3**	**2/3**	**MPUAB**	**S**	**Hypothetical protein**
NP_757879	**6/1**	**1/2**	**1/7**	**MPHF2**	**S**	**Hypothetical protein**
NP_853031	**2/2**	**1/1**	**1/1**	**MGR**	**S**	**Hypothetical protein**
NP_853193	**1/6**	**1/1**	**4/5**	**MGR**	**M (1)**	**Hypothetical protein**
YP_015730	**1/2**	**1/1**	**2/1**	**MM163K**	**S**	**Hypothetical protein**
NP_975752	1/1	5/1		MMSC	S	Conserved hypothetical prolipoprotein
NP_325964	1/2	1/1		MPUAB	s	Hypothetical protein
NP_326044	1/2	6/1		MPUAB	M (2)	Hypothetical protein
NP_758027	1/1	1/1		MPHF2	S	Hypothetical protein
YP_016270	1/1	1/1		MM163K	M (1)	Hypothetical protein
YP_016309	1/2	1/1		MM163K	S	Hypothetical protein
NP_853186	1/1	2/1		MGR	M (1)	Hypothetical protein
AAD10542	1/2		3/2	MG	M (1)	Hypothetical protein
AAD12372	1/1		1/1	MG	S	Hypothetical protein
NP_072670	3/2		1/4	MGG37	S	Hypothetical protein
NP_072802	1/2		1/1	MGG37	S	Hypothetical protein
NP_073131	1/3		1/1	MGG37	S	Hypothetical protein
NP_109841	1/2		2/1	MPM129	S	Hypothetical protein
NP_325873	1/1		1/1	MPUAB	S	Hypothetical protein
NP_326247	1/1		2/1	MPUAB	M (1)	Hypothetical protein
NP_326295	1/1		3/4	MPUAB	M (1)	Hypothetical protein
NP_326548	1/1		1/1	MPUAB	M (1)	Hypothetical protein
NP_326586	1/3		1/1	MPUAB	M (1)	Hypothetical protein
NP_757765	2/3		5/3	MPHF2	M (2)	Hypothetical protein
NP_757965	1/1		1/2	MPHF2	S	Hypothetical protein
NP_758125	1/4		6/1	MPHF2	S	Hypothetical protein
NP_758126	1/4		6/1	MPHF2	S	Hypothetical protein
NP_852801	1/1		2/1	MGR	S	Hypothetical protein
NP_853043	1/1		1/1	MGR	S	Hypothetical protein
NP_853113	1/2		2/3	MGR	S	Hypothetical protein
NP_975637	1/2		3/1	MMSC	S	Hypothetical protein
S05299	1/4		6/2	MHY	S	Hypothetical protein
Mobile and extrachromosomal element functions						
CAD60178	2/1		4/1	MB	S	Transposase
Protein fate						
NP_758282	**1/3**	**2/1**	**3/3**	**MPHF2**	**S**	**Leucyl aminopeptidase**
NP_110261	1/3		3/3	MPM129	S	Leucyl aminopeptidase
YP_015871	2/2		1/2	MM163K	S	Leucyl aminopeptidase
YP_016160	3/2		4/2	MM163K	S	Leucyl aminopeptidase
NP_326557	1/2		3/2	MPUAB	M (2)	Leucyl aminopeptidase
CAB96373	1/1		1/2	MAG	S	Heat shock protein
CAB96377	1/1		1/2	MB	S	Heat shock protein
NP_326350	**3/6**	**3/2**	**9/7**	**MPUAB**	**S**	**Heat shock ATP-dependent protease**
YP_015965	1/3		2/2	MM163K	S	ATP-dependent Lon protease
Protein synthesis						
CAA39292	**7/25**	**2/16**	**23/30**	**MP**	**S**	**Translation elongation factor Tu (EF-Tu)**
NP_073121	**8/23**	**2/15**	**22/29**	**MGG37**	**S**	**Translation elongation factor Tu (EF-Tu)**
NP_110354	**7/25**	**2/16**	**23/30**	**MPM129**	**S**	**Translation elongation factor Tu (EF-Tu)**
YP_015921	**25/86**	**5/4**	**82/68**	**MM163K**	**S**	**Translation elongation factor Tu (EF-Tu)**
CAC87988	3/2		4/3	MMSC	S	Translation elongation factor Tu (EF-Tu)
NP_326236	16/44		55/14	MPUAB	S	Translation elongation factor Tu (EF-Tu)
JH0416	10/35		27/26	MHO	S	Translation elongation factor Tu (EF-Tu) precursor
NP_326259	4/7		11/5	MPUAB	S	Translation elongation factor G (EF-G)
YP_016064	3/4		6/2	MM163K	S	Translation elongation factor G (EF-G)
NP_757417	1/2		3/4	MPHF2	S	Translation elongation factor G (EF-G)
NP_326344	1/1		1/1	MPUAB	S	Translation elongation factor P (EF-P)
NP_072838	1/2		3/2	MGG37	M (1)	30S ribosomal protein S13
NP_326415	6/8		21/3	MPUAB	S	30S ribosomal protein sl9
NP_975715	1/1		1/2	MMSC	S	30S ribosomal protein S3
CAA29710	1/1		1/2	MC	S	30S ribosomal protein S3

Table 1 (*Continued*)

Protein ID	Peptide hits (PH1/PH2)[a]			Species[b]	Location[c]	Gene function
	T	M	C			
YP_015949	1/2		1/4	MM163K	S	30S ribosomal protein s5
NP_326401	1/2		1/3	MPUAB	S	30S ribosomal protein s5
NP_326260	**1/2**	**1/1**	**2/1**	**MPUAB**	**S**	**30S ribosomal protein S7**
YP_015927	3/3		2/1	MM163K	S	30S ribosomal protein s9
NP_975720	1/1	3/2		MMSC	S	50S ribosomal protein L4
NP_326406	3/1		4/3	MPUAB	S	50S ribosomal protein L5
YP_015947	1/13		18/3	MM163K	S	50S ribosomal protein 16
YP_016136	**1/1**	**1/2**	**2/2**	**MM163K**	**S**	**Alanine-tRNA ligase**
S77844	1/1		1/1	MC	S	Alanine-tRNA ligase
NP_975181	1/1		1/1	MMSC	S	Alanine-tRNA ligase
NP_975566	2/1	1/1	2/1	MMSC	S	Isoleucine-tRNA ligase
NP_326088	1/1		2/2	MPUAB	S	Valine-tRNA ligase
YP_015855	**5/4**	**3/2**	**3/4**	**MM163K**	**M (1)**	**Valyl-tRNA synthetase**
NP_072959	1/1		2/1	MGG37	S	Alanyl-tRNA synthetase (alaS)
CAA83717	1/1		1/1	MC	S	Ala-tRNA synthetase
AAL86923	2/2		6/5	MGAL	S	Glutamyl-tRNA synthetase
YP_015741	4/5		8/3	MM163K	S	Lysyl-tRNA synthetase
NP_072845	2/1		3/1	MGG37	S	Pseudouridylate synthase I (hisT)
NP_757499	2/2		1/2	MPHF2	S	Translation initiation factor IF-2
NP_758420	1/1		1/1	MPHF2	S	Dimethyladenosine transferase
YP_016073	1/1		3/2	MM163K	S	Glutamyl-tRNA amidotransferase subunit B
Purines, pyrimidines, nucleosides, and nucleotides						
NP_326497	1/3		5/1	MPUAB	S	CTP synthase (UTP-ammonia ligase)
YP_016233	1/2		2/1	MM163K	S	Pyrimidine (thymidine)-nucleoside phosphorylase
Transcription						
YP_015847	1/1		1/1	MM163K	S	Transcription elongation factor NusA
AAP20120	2/1		3/2	MB	S	RNA polymerase beta subunit
NP_757465	1/1		1/1	MPHF2	S	DNA-directed RNA polymerase subunit beta
NP_757466	1/2		3/1	MPHF2	S	DNA-directed RNA polymerase subunit beta'
NP_853171	2/2		1/1	MGR	S	RNA polymerase sigma factor (RpoD)
Transport and binding proteins						
NP_325857	**2/9**	**6/1**	**9/6**	**MPUAB**	**S**	**ABC transporter ATP-binding protein**
NP_325953	2/1	2/2		MPUAB	S	ABC transporter ATP-binding protein
NP_975694	1/1	3/1		MMSC	S	ABC transporter, ATP-binding component
NP_326545	2/1		1/2	MHY	S	ABC transporter ATP-binding protein (P115-like)
NP_975462	**1/2**	**1/1**	**2/1**	**MMSC**	**S**	**P115-like protein with structural maintenance of chromosomes (SMC_C) motif**
NP_975389	**1/1**	**2/2**	**1/2**	**MMSC**	**S**	**Na+ ABC transporter, ATP-binding component**
NP_758263	1/1		1/1	MPHF2	S	Oligopeptide ABC transporter ATP-binding protein
NP_975187	3/1		1/2	MMSC	S	Oligopeptide ABC transporter, substrate-binding component
CAA68077	1/5	1/1		MH	S	Oligopeptide transport ATP-binding protein homolog
NP_110316	1/1		1/1	MPM129	S	PEP-dependent HPr protein kinase phosphoryltransferase (Enzyme I)
NP_975471	1/1		1/1	MMSC	M (1)	Phosphate ABC transporter, substrate-binding component
NP_852862	2/4		2/3	MGR	S	Phosphoenolpyruvate-protein kinase (ptsA)
NP_073083	1/1		1/1	MGG37	S	Phosphate ABC transporter, ATP-binding protein (pstB)
NP_852823	**1/1**	**1/1**	**2/2**	**MGR**	**S**	**Phosphate ABC transporter, ATP-binding protein (pstB)**
NP_757975	4/2		2/1	MPHF2	S	Sugar ABC transporter ATP-binding protein
NP_326450	1/2		1/1	MPUAB	S	Sugar ABC transporter ATP-binding protein MGLA
NP_326175	1/1		4/1	MPUAB	S	Xylose ABC transporter ATP-binding protein
NP_975044	1/1		4/2	MMSC	M (8)	Predicted permease
NP_109922	1/1		1/2	MPM129	S	Membrane export protein family
Unclassified						
NP_110298	1/1		1/2	MPM129	S	Phosphate specific
NP_326214	2/1		1/2	MPUAB	M (2)	TRSE-like protein (with ATP/GTP-binding motif)
Unknown function						
NP_975326	1/2		1/2	MMSC	S	Ribonuclease H-related protein
AAC45345	2/1		4/1	MF	M (9)	Putative polytopic protein
NP_757542	2/4		2/5	MPHF2	S	Predicted cytoskeletal protein
NP_757818	2/1		2/1	MPHF2	M (4)	Putative integral membrane protein
NP_975356	3/5		8/4	MMY	M (1)	Conserved hypothetical transmembrane protein
NP_975600	1/1		2/1	MMY	M (3)	Conserved hypothetical transmembrane protein
CAC95143	3/2		5/3	MHO	S	Variable membrane protein (vmp)
CAF32691	1/3	3/1		MHO	S	Variable membrane protein (vmp) precursor
CAD67982	1/2		2/1	MHO	S	Variable membrane protein (vmp) precursor
CAF32691	1/3		1/3	MHO	S	Variable membrane protein (vmp) precursor
AAT27959	1/1		2/1	MM163K	M (3)	Unknown function, identified in proteogenomic mapping

Table 1 (*Continued*)

Protein ID	Peptide hits (PH1/PH2)[a]			Species[b]	Location[c]	Gene function
	T	M	C			
YP_016315	1/1		1/3	MM163K	S	Predicted hydrolase
YP_016164	3/3		1/2	MM163K	S	COF family HAD hydrolase protein

[a] Peptide hits from 2D MS/MS data analysis. T, M, and C represent total protein, membrane phase and cytosolic phase, respectively. Each phase has two replicates. The protein identified in TM are gray-highlighted and the ones in TMC are highlighted in bold. PH1/PH2 denotes the peptide hits from biological replicate 1 and the peptide hits from biological replicate 2.

[b] *Mycoplasma* species has the protein identified. MA: *Mycoplasma arthritidis*; MB: *Mycoplasma bovis*; MC: *Mycoplasma capricolum*; MF: *Mycoplasma fermentans*; MGAL: *Mycoplasma gallinarum*; MGG37: *Mycoplasma genitalium* G-37; MGR: *Mycoplasma gallisepticum* R ; MHO: *Mycoplasma hominis* ; MHY: *Mycoplasma hyopneumoniae* ; MM163K: Mycoplasma mobile 163K; MM: *Mycoplasma mycoides*; MMSC: *Mycoplasma mycoides* subsp. mycoides SC str. PG1; MPHF2: *Mycoplasma penetrans* HF′-2; MPUAG: *Mycoplasma pulmonis* UAB CTIP; MHO: *Mycoplasma hominis*; MPM129: *Mycoplasma pneumoniae* M1 29.

[c] Location is predicted by SOSUI. S: solutionable protein; M: membrane protein. The predicted helix number for each membrane protein is also listed in the parenthesis.

[d] This is an undefined mycoplasma species.

rates are evident for all amino acids (Matsui *et al.*, 2006). *M. gallinarum* is a non-fermentative and arginine-utilizing mycoplasma (Aluotto et al., 1972). Its growth requires 13 amino acids, including Ala, Arg, Asp, Cys, Glu, Gly, His, Ilu, Leu, Met, Phe, Thr, and Tyr (Lund and Shorb, 1966). As with other non-fermentative mycoplasmas, arginine can be utilized by *M. gallinarum* as an energy source via the arginine dihydrolase pathway. The potential of multiple copies of leucyle aminopeptidases in *M. gallinarum* suggest the important roles of aminopeptidase gene in supplying the required nutrients. It is interesting that the leucine aminopeptidase we characterized earlier has not been identified in this study (Wan et al., 2004a). It is not clear whether there is a post-translation modification, which may lead to this mis-identification. It is worth mentioning that, without the genomic sequence of *M. gallinarum*, it is even more difficult to determine exactly the number of aminopeptidase genes in *M. gallinarum*.

The presence of possible multiple copies of leucyle aminopeptidases suggest *M. gallinarum* might have a more flexible nutrient acquisition pathway than some other host-specific mycoplasmas. For instance, *M. genitalium* has one methionine aminopeptidase gene and one leucyl aminopidase gene; *M. gallisepticum* only has one leucyl aminopeptidase. It is interesting that previous studies in *A. laidlawii*, *M. bovirhinis*, *M. bovis*, and *M. dispar* showed that aminopeptidase activity in mycoplasmas was negatively correlated with their pathogenesis, meaning more aminopeptidase activity might result in lower pathogenicity (Neill and Ball, 1980). Thus, *M. gallinarum* aminopeptidases may provide a useful model for determining the role of aminopeptidases in host colonization and pathogenesis.

We also identified two pyruvate dehydrogenase E1-beta component proteins, a phosphoenolpyruvate carboxylase, a phosphopyruvate hydratase, and a glucose-6-phosphate isomerase in *M. gallinarum*. This supports the utilization of organic acid, such as ethanol, L-lactate, pyruvate, or 2-oxobutyrate, as alternative energy sources in *M. gallinarum* (Taylor et al., 1994). It was also reported that the organic acid catabolism does not interfere with arginine hydrolysis in *M. gallinarum* (Taylor et al., 1994).

Mycoplasmas are rich in transporter genes, which can be categorized into at least nine transporter families (Glass et al., 2000). *M. gallinarum* has a large number of ATP synthase proteins, as is similar to many other mycoplasmas (Table 1). In addition, similar to other mycoplasmas, various types of transporter or associated genes are found in our analyses, including Na^+ ABC transporter, oligopeptide ABC transporter ATP-binding protein, phosphate ABC transporter, sugar ABC transporter ATP-binding protein, and the xylose ABC transporter ATP-binding protein. The data also suggest this genome has a phosphoenolpyruvate-dependent sugar phosphotransferase transport system (PTS) since PEP-dependent HPr protein kinase phosphoryltransferase was identified. Because mycoplasmas do not encode many of the major genes for synthesis of macromolecule precursors such as amino acids, lipids, and nucleotides, the survival of mycoplasmas depends on a nutrient rich environment (Razin et al., 1998). The need to transport nutrients scavenged from the host environment underscores the role that the mycoplasma membrane proteins have in metabolism.

In summary, we characterized the protein profiling of *M. gallinarum* using applied 2D LC–MS/MS. Our results suggested that *M. gallinarum* has a unique protein profiling in cell adhesion and nutrition acquisition. *M. gallinarum* possesses homologs of cytadhesin proteins found in other mycoplasmas lacking an organized tip organelle. The presence of multiple copies of aminopeptidases, organic acid catabolism associated proteins, and a large amount of transporter proteins in *M. gallinarum* would potentially allow this mycoplasma to adapt better in different hosts. Through genomic sequencing and functional analysis, future studies will further characterize the roles of these proteins in colonization and host adaptation properties of *M. gallinarum*.

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